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Concise procedure for the synthesis of cardiolipins having different fatty acid combinations

Masato Abe, Shigeki Kitsuda, Shunji Ohyama, Shinya Koubori, Masatoshi Murai, Hideto Miyoshi *

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

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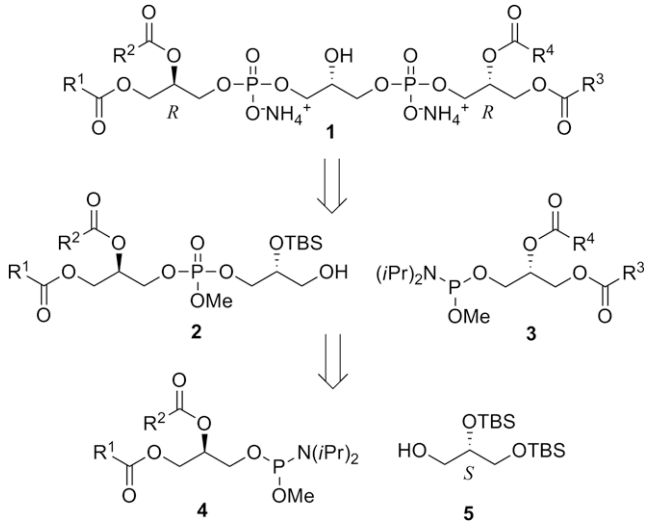
ABSTRACT

Production of a wide variety of cardiolipin (CL) analogues is critical for studying molecular mechanism of diverse biological functions of CL in mitochondria. We describe a concise procedure for the synthesis of CL using a phosphoramidite approach, which allows for the production of diverse CL analogues bearing linoleic acid(s) at any position on the glycerol backbone.

Cardiolipin (CL, **1** in Scheme 1), a negatively charged phospholipid bearing four fatty acid chains, is a major phospholipid found in mammalian mitochondria (up to 20–25%) with a multitude of biological functions (reviewed in Refs. 1,2). For instance, CL is responsible for regulation of the activity of several mitochondrial enzymes involved in ATP biosynthesis,[3,4] though the precise molecular mechanism of its regulation remains to be elucidated. Recently, Kagan and co-workers demonstrated that in the early stages of apoptosis, cytochrome *c* (cyt *c*) bound to the CL-containing mitochondrial inner membrane acts as a peroxidase that selectively catalyzes CL peroxidation: this event contributes to the release of cyt *c* into the cytosol, and initiation of the apoptotic program.[5,6] Although the fatty acid composition of CL is suggested to be critical for the formation of the cyt *c*-CL complex to yield the peroxidase activity,[6] the molecular mechanism has not been sufficiently studied. This is primarily because CL analogues used in the previous biochemical studies are limited to natural and/or a few commercially available CL analogues; in the former, the chain moiety is a mixture of various fatty acids, and in the latter, the chemical variation of fatty acid chains is very poor. Therefore, to explore in detail the molecular mechanisms of both the formation of the cyt *c*-CL complex and the induction of peroxidase activity of cyt *c*, biochemical studies using structurally variable CL analogues are needed.

Several procedures for the synthesis of CL have been reported.[7] Previous studies however were not necessarily concerned with generating structurally diverse CL analogues. For example, some procedures (e.g., Ref. 7e) for the synthesis of CL bearing only saturated fatty acid chains are not suitable for the synthesis of CL containing linoleic acid(s) (C18:2), which is a major fatty acid of natural CL in mammalian mitochondria,[2] because the *cis*-1,4-diene structure in linoleic acid is remarkably degradable under the conditions. In addition, some methods are not feasible for the routine preparation of large quantities owing to use of highly unstable intermediates or expensive reagents (e.g., Refs. 7c,d).

The phosphoramidite approach, widely exploited in oligonucleotide chemistry (e.g., Ref. 8), described by Ahmad and colleagues is an excellent way to obtain large quantities of CL analogues in high yields.[9] Unfortunately, they did not use linoleic acid as the acyl chain(s), and their procedure does not give asymmetrically substituted CL analogues. We herein describe a concise procedure for the synthesis of CL using phosphoramidite chemistry, which produces diverse CL analogues bearing linoleic acid(s) at any position of the four acyl chains on a gram scale. This approach also allows for the production of CL containing a biophysical probe (nitroxide spin-label, fluorescent label, etc.) in one of the four chains.



Scheme 1. Retrosynthetic analysis of cardiolipin bearing four different acyl chains. R^1–R^4: fatty acids.

* Corresponding author. Tel.: +81 75 753 6119; fax: +81 75 753 6408.
E-mail address: miyoshi@kais.kyoto-u.ac.jp (H. Miyoshi).

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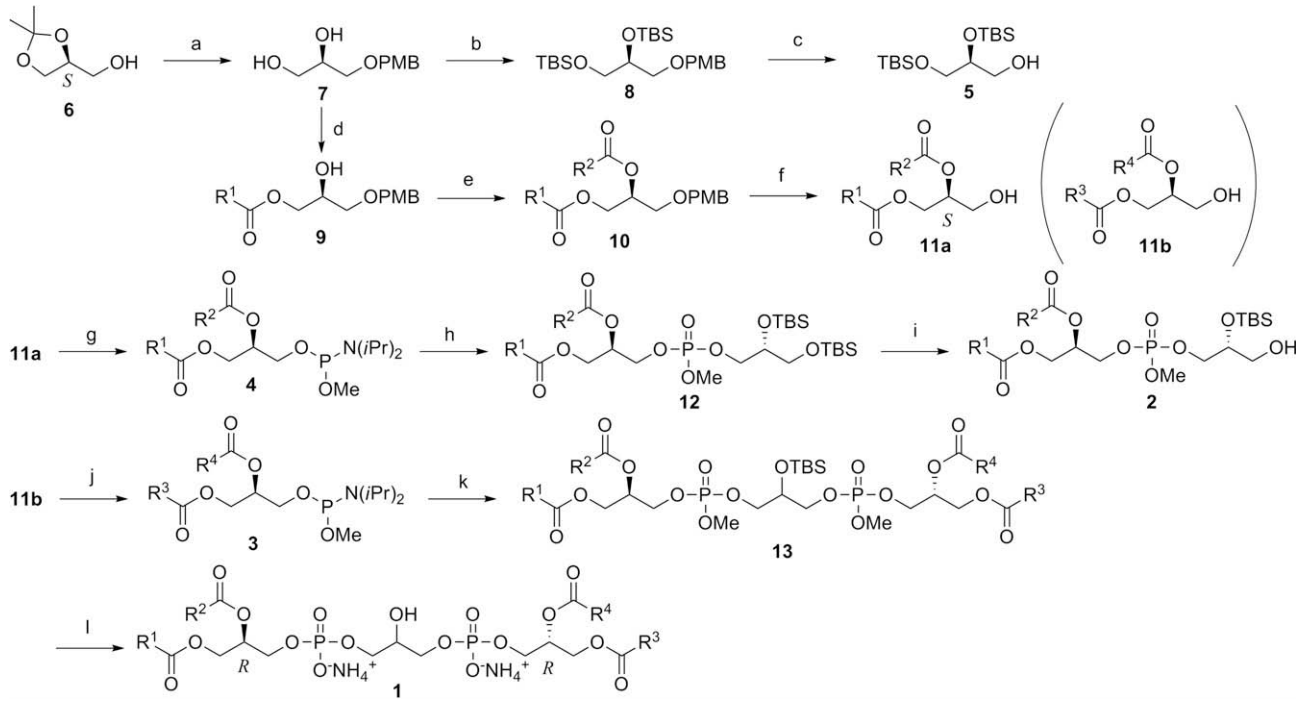
Retrosynthetic analysis of CL (**1**) bearing four different acyl chains (R^1–R^4) gives two phosphoramidite fragments, **3** and **4**, and a diprotected glycerol fragment, **5** (Scheme 1). The choice of protection groups for alcohol and phosphate functionalities is critical in the synthesis of CL bearing an unsaturated acyl chain(s). To find milder reaction conditions for avoiding the decomposition of an unsaturated acyl chain(s), we introduced a sensitive linoleic acid into all four positions, and carefully checked the *cis*-1,4-diene structure by ^{1}H and ^{13}C NMR spectroscopy. All procedures for the reaction and purification were performed with shielding from light.

PMB protection of the commercially available (*S*)-1,2-*O*-isopropylidene-*sn*-glycerol **6** followed by acid-catalyzed hydrolysis of the acetal afforded, in good isolated yield, diol **7**,[7e,10] a key intermediate for all three fragments (Scheme 2). DCC-mediated esterification of **7** with 0.4 equiv of linoleic acid selectively afforded the monoester **9** without migration of the acyl group, and no reacted **7** was recycled. Subsequent esterification of **9** with linoleic acid (1.2 equiv) quantitatively afforded the diester **10**. Using desired fatty acids in place of linoleic acid, the diester having different acyl chains in the *sn*-1- and *sn*-2-glycerol positions can be prepared. It is noteworthy that installation of the alkyl group into the glycerol building block is extremely difficult once the phosphate moiety has been constructed.[10]

Removal of the protection group of the *sn*-3 position in 1,2-diacyl-*sn*-glycerol is a delicate step for the synthesis of phospholipids because of migration of the acyl group from the *sn*-2 position to the *sn*-3 position during the reaction or isolation conditions.[10–12] The acyl chain migration can be determined accurately by ^{1}H NMR.[10–12] Through careful examination of several reaction conditions, we found that the treatment of **10** with 10 equiv of CAN in CH_3CN/H_2O (9:1, v/v) at room temperature affords **11a** in high yield without migration of the acyl group and the decomposition of the *cis*-1,4-diene structure. Removal of the PMB group with the frequently used reagent DDQ in wet CH_2Cl_2 (0.5% water in volume) at room temperature resulted in low yields of the product (30–40%). Attempts to improve the yield were unsuccessful and only led to partial decomposition of the double bonds. We also examined the protection of **6** with a silyl group (TBS or TBDPS) and its deprotection from the corresponding 1,2-di-*O*-acyl-3-*O*-silylated glycerol with TBAF, whereas significant migration after the deprotection occurred under the basic conditions.[10]

Coupling of the 1,2-di-*O*-acyl-*sn*-glycerol **11a** with bifunctional *N*,*N*-diisopropylmethylphosphonamidic chloride in the presence of DIPEA (1.2 equiv) afforded the 1,2-diacylglyceryl (*N*,*N*-diisopropylamino)phosphoramidite **4**, which was used as such for the next reaction without any purification. Subsequent coupling of **4** with the TBS-protected glycerol **5** in the presence of 1*H*-tetrazole (1.2 equiv) as a promoter of the reaction[8] gave a phosphite triester intermediate, which was oxidized in situ with nBu$_4$NIO$_4$ at −20 °C to afford intermediate **12** in moderate yield after column purification. Oxidation by the frequently used reagent *t*-BuOOH[9,10,12] resulted in slight, but not negligible, decomposition of the double bonds in the acyl chains. Treatment of **12** with HF-pyridine/pyridine/dry-THF (1:2:5, v/v) for 1.5 h at 0 °C selectively removed the TBS group of the primary alcohol to afford the phosphatidylglycerol methyl ester **2** in 63% yield along with a byproduct (20%) due to cleavage of both TBS groups; however, this byproduct was recycled through reprotection with TBSCl to give **12**. Using differently protected glycerol (2-*O*-Bn-3-*O*-TBS-(2*S*)-*sn*-glycerol and 3-*O*-TBS-2-*O*-PMB-(2*S*)-*sn*-glycerol) in place of **5**, we actually prepared the corresponding phosphatidylglycerol methyl esters, and examined the sequential procedures. As a result, the reason for selecting the TBS group to protect the secondary alcohol in **12** is due to its satisfactory removal in a final reaction step l, as described later.



Scheme 2. Reagents and conditions: (a) (i) PMBCl, NaH, DMF, rt, 2 h; (ii) AcOH/H_2O (4:1, v/v), 50 °C, 2 h, 85% (two steps); (b) TBSCl, imidazole, CH_2Cl_2, 70 °C, 2.5 h, 97%; (c) DDQ, CH_2Cl_2/H_2O (20:1, v/v), rt, 30 min, 88%; (d) R^1COOH (0.4 equiv), DCC, DMAP, CH_2Cl_2, 0 °C, 6 h, 84% (65% of **7** recovered); (e) R^2COOH (1.2 equiv), DCC, DMAP, CH_2Cl_2, rt, 2.5 h, 99%; (f) CAN (10 equiv), CH_3CN/H_2O (9:1, v/v), rt, 1.5 h, 93%; (g) (*i*-Pr)$_2$NP(OMe)Cl (1.2 equiv), DIPEA (1.2 equiv), CH_2Cl_2, rt, 1 h; (h) (i) **5**, 1*H*-tetrazole (1.2 equiv), CH_3CN, rt, 2.5 h; (ii) Bu_4NIO_4, CH_2Cl_2, −20 °C to rt, 2 h, 70% (three steps); (i) HF-pyridine/pyridine/THF (1:2:5), rt, 1 h, 63%; (j) (*i*-Pr)$_2$NP(OMe)Cl (1.2 equiv), DIPEA (1.2 equiv), CH_2Cl_2, rt, 1 h; (k) (i) **2**, 1*H*-tetrazole (1.2 equiv), CH_3CN, rt, 2.5 h; (ii) Bu_4NIO_4, CH_2Cl_2, −20 °C to rt, 2 h, 55% (three steps); (l) (i) NaI (2.5 equiv), 2-butanone, reflux, 1 h; (ii) 1.0 M HCl/H_2O/THF (0.1:1:2, v/v), rt, 13 h, then 25% NH_4OH aq, 71% (two steps).

With half of the CL molecule (**2**) in hand, the whole molecule is constructed as follows. The desired 1,2-diacyl-*sn*-glycerol (**11b**) prepared by the method described above was reacted with *N*,*N*-diisopropylmethylphosphonamidic chloride in the presence of DIPEA to give 1,2-diacyl-*sn*-glyceryl (*N*,*N*-diisopropylamino)phosphoramidite (**3**). To this intermediate was added a CH_2Cl_2 solution of **2** in the presence of 1*H*-tetrazole, followed by in situ oxidation with nBu$_4$NIO$_4$ at −20 °C to afford a phosphotriester of the CL precursor **13** in a moderate yield. Deprotection of the phosphate function in **13** has to be carried out prior to the removal of the TBS group to avoid migration of the phosphate.[9d] Therefore, the methyl groups in **13** were cleaved with sodium iodide (2.5 equiv) in refluxing 2-butanone. Finally, removal of the TBS group was accomplished by treating the resultant phosphate in 1.0 M HCl/THF/H_2O (0.1:2:1, v/v) without any decomposition of the double bonds, and converted into the ammonium salt **1** by treatment with 0.1 M HCl followed by 25% ammonium hydroxide.[9c,13] For the synthesis of unsaturated CL [tetraoleoyl (C18:1)-CL], Ahmad and co-workers used a levulinoyl group as a protection group of the secondary alcohol of the glycerol bridge in the CL precursor,[9a,d] corresponding to **13** in our study, and performed deprotection of this group by hydrazinolysis[14] with hydrazine in pyridine. The application of this method, however, to the demethylated **13** resulted in about 10% decomposition of the double bonds.

CL contains two 1,2-diacyl-*sn*-glycero-3-phosphoryl moieties linked by a glycerol bridge, one phosphatidyl moiety is in *pro-R* and the other is in *pro-S* position with respect to the central carbon atom of the glycerol bridge. Obviously, the central carbon atom becomes a true chiral center if the two phosphatidyl residues contain different fatty acids.

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13. *The data for* **1**: FTIR (ATR): 3211, 3009, 2925, 2854, 1739, 1457, 1377, 1211, 1064 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 5.39–5.28 (m, 16H), 5.23 (m, 2H), 4.45 (dd, J = 12.0, 3.1 Hz, 2H), 4.19 (dd, J = 12.1, 6.9 Hz, 2H), 3.99 (dd, J = 5.5, 5.5 Hz, 4H), 3.95–3.88 (m, 5H), 3.60 (br s, 1H), 2.77 (t, J = 6.3 Hz, 8H), 2.34 (t, J = 7.4 Hz, 4H), 2.31 (t, J = 7.4 Hz, 4H), 2.06 (dt, J = 7.0, 7.0 Hz, 16H), 1.68–1.56 (m, 8H), 1.43–1.26 (m, 56H), 0.91 (t, J = 6.9 Hz, 12H); ^{13}C NMR (125 MHz, CD_3OD) δ 173.50 (2C), 173.18 (2C), 129.64 (4C), 129.57 (4C), 127.82 (4C), 127.25 (4C), 78.16 (2C), 70.80, 66.32 (2C), 63.44 (2C), 62.43 (2C), 33.70 (2C), 33.55 (2C), 31.39 (4C), 29.48 (4C), 29.20 (4C), 29.13 (4C), 29.08 (4C), 28.99 (4C), 28.92 (4C), 26.90 (4C), 25.29 (4C), 24.71 (4C), 22.35 (4C), 13.19 (4C); ^{31}P NMR (162 MHz, CD_3OD) δ 0.94 (2P); HRMS (ESI) calcd for $C_{81}H_{141}O_{17}P_2$ $(M-2NH_3-H)^-$ 1447.9649; found 1447.9594, calcd for $C_{81}H_{140}O_{17}P_2$ $(M-2NH_3-2H)^{2-}$ 723.4788; found 723.4750.
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